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(54) Title: CHROMATOGRAPHY LIGAND

(57) Abstract: The present invention relates to a chromatography ligand defined by the following formula (I) $R_1-R_2-N(R_3)-R_4-R_5$, wherein R_1 is a substituted or non-substituted phenyl group; R_2 is a hydrocarbon chain comprising 0-4 carbon atoms; R_3 is a hydrocarbon chain comprising 1-3 carbon atoms; R_4 is a hydrocarbon chain comprising 1-5 carbon atoms; and R_5 is OH or H. The invention also comprises a separation matrix, comprising the described ligands coupled to a porous support, such as particles or a membrane. The ligand and matrix according to the invention is useful for purification of biomolecules or organic compounds, such as proteins, polypeptides, DNA etc. An advantageous use according to the invention is the purification of antibodies.

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CHROMATOGRAPHY LIGAND

Technical field

The present invention relates to novel chromatography ligands, which are useful for the purification of biomolecules such as proteins. The present ligands are useful e.g. for the purification of antibodies, preferably immobilised to a porous support such as particles or a membrane. Consequently, the invention also encompasses a chromatography matrix comprising the novel ligands, a method for the preparation thereof and a kit for the purification of antibodies.

Background

The immune system is composed of many interdependent cell types that collectively protect the body from bacterial, parasitic, fungal, viral infections and from the growth of tumour cells. The guards of the immune system are macrophages that continually roam the bloodstream of their host. When challenged by infection or immunisation, macrophages respond by engulfing invaders marked with foreign molecules known as antigens. This event, mediated by helper T cells, sets forth a complicated chain of responses that result in the stimulation of B-cells. These B-cells, in turn, produce proteins called antibodies, which bind to the foreign invader. The binding event between antibody and antigen marks the foreign invader for destruction via phagocytosis or activation of the complement system. A number of different classes of antibodies, also known as immunoglobulins, exist, such as IgA, IgD, IgE, IgG, and IgM. They differ not only in their physiological roles but also in their structures. From a structural point of view, IgG antibodies have been extensively studied, perhaps because of the dominant role they play in a mature immune response. Polyclonal antibodies are produced according to standard methods by immunisation of an animal with the appropriate antigen. In response, the animal will produce antibodies which are polyclonal. However, for many purposes, it is desired to have a single clone of a certain antibody, known as monoclonal antibodies. Monoclonal antibodies (MAbs) are produced by hybrid or fused cells comprised of a fusion between a normal B-cell, which produces only a single

antibody, to an abnormal myeloma tumour cell. The resulting hybrid, known as a hybridoma, is these days used in standard methods for the production of antibodies.

5 The biological activity that the immunoglobulins possess is today exploited in a range of different applications in the human and veterinary diagnostic, health care and therapeutic sector. In fact, in the last few years, monoclonal antibodies and recombinant antibody constructs have become the largest class of proteins currently investigated in clinical trials and receiving FDA approval as therapeutics and diagnostics. Complementary to expression systems and production strategies, efficient purification protocols are
10 required to obtain highly pure antibodies in a simple and cost-efficient manner.

Traditional methods for isolation of immunoglobulins are based on selective reversible precipitation of the protein fraction comprising the immunoglobulins while leaving other groups of proteins in solution. Typical precipitation agents are ethanol, polyethylene
15 glycol, lyotropic salts such as ammonium sulphate and potassium phosphate, and caprylic acid. Typically, these precipitation methods are giving very impure products while at the same time being time consuming and laborious. Furthermore, the addition of the precipitating agent to the raw material makes it difficult to use the supernatant for other purposes and creates a disposal problem, which is particularly relevant when
20 speaking of large-scale purification of immunoglobulins.

An alternative method for isolation of immunoglobulins is chromatography, which embraces a family of closely related separation methods. The feature distinguishing chromatography from most other physical and chemical methods of separation is that
25 two mutually immiscible phases are brought into contact wherein one phase is stationary and the other mobile. The sample mixture, introduced into the mobile phase, undergoes a series of interactions with the stationary and mobile phases as it is being carried through the system by the mobile phase. Interactions exploit differences in the physical or chemical properties of the components in the sample. These differences govern the rate
30 of migration of the individual components under the influence of a mobile phase moving through a column containing the stationary phase. Separated components emerge in the

order of increasing interaction with the stationary phase. The least retarded component elutes first, the most strongly retained material elutes last. Separation is obtained when one component is retarded sufficiently to prevent overlap with the zone of an adjacent solute as sample components elute from the column. Efforts are continuously being made to design the optimal stationary phase for each specific separation purpose. Such a stationary phase is commonly comprised of a support or base matrix to which a ligand comprising functional i.e. binding groups has been attached. Reference is commonly made to each kind of chromatography based on the principle of interaction it utilises, such as affinity chromatography, hydrophobic interaction chromatography and ion-exchange chromatography.

Affinity chromatography is based on specific interactions between a target biomolecule and a biospecific ligand according to a principle of lock-key recognition. Thus, the target and ligand will constitute an affinity pair, such as antigen/antibody, enzyme/receptor etc. Protein-based affinity ligands are well known, such as Protein A and Protein G affinity chromatography which are both widespread methods for isolation and purification of antibodies. It is well known that Protein A chromatography provides an outstanding specificity, particularly towards monoclonal antibodies, and consequently high purities are obtainable. Used in combination with ion exchange, hydrophobic interaction, hydroxyapatite and/or gel filtration steps, Protein A-based methods have become the antibody purification method of choice for many biopharmaceutical companies, see e.g. WO 8400773 and US 5,151,350. However, due to the peptide bonds of the proteins, protein A matrices present a certain degree of alkaline sensitivity. In addition, when Protein A matrices are used to purify antibodies from cell culture media, proteases originating from the cells may cause leakage of Protein A, or peptide fragments thereof.

Ion exchange chromatography is frequently used in protocols for the isolation of immunoglobulins. In anion exchange chromatography, negatively charged amino acid side chains of the immunoglobulin will interact with positively charged ligands of a chromatography matrix. In cation exchange chromatography on the other hand,

positively charged amino acid side chains of the immunoglobulin will interact with negatively charged ligands of a chromatography matrix.

Hydrophobic interaction chromatography (HIC) is another method described and used in protocols for the isolation of immunoglobulins. If a highly pure immunoglobulin product is the object, it is commonly recommended to combine HIC with one or more further steps. In HIC, in order to make the immunoglobulin bind efficiently to the HIC matrix, addition of lyotropic salts to the mobile phase is required. The bound immunoglobulin is subsequently released from the matrix by lowering the concentration of lyotropic salt.

Thus, a disadvantage of this procedure is the necessity to add lyotropic salt to the raw material, as this may cause problems and a consequently increased cost to the large-scale user. For example, for raw materials such as whey, plasma, and egg yolk, the addition of lyotropic salts to the raw materials would in many instances be prohibitive in large-scale applications, as the salt could prevent any economically feasible use of the immunoglobulin depleted raw material. An additional problem in large-scale applications would be the disposal of several thousand litres of waste.

US 5,945,520 (Burton et al) discloses mixed mode chromatographic resins which exhibit a hydrophobic character at the pH of binding and a hydrophilic and/or electrostatic character at the pH of desorption. The resin is specifically designed to bind the target compound from an aqueous solution at both a low and high ionic strength. This is achieved by selected ionisable ligands comprising a spacer arm and at least one ionisable functionality, wherein the density of the ionisable ligands on the solid support matrix is greater than the smaller of either about 150 $\mu\text{mol/mL}$ resin or 1 mmol/gram dry weight of resin. In addition, the hydrophobic character of the resin comprising said ionisable ligands is sufficient to bind at least 50% of the target compound in an aqueous medium at high and low ionic strength at a first pH. Illustrative examples of the ionisable functionality are 4-(aminomethyl)pyridine, 3-(aminomethyl)pyridine, 2-(aminomethyl)pyridine, 1-(3-aminopropyl)-imidazole, 2-(aminomethyl)-benzimidazole, 4-(3-aminopropyl)morpholine.

Further, WO 01/38228 (Belew et al.) relates to a method for anion-exchange adsorption wherein thioether anion-exchangers are used to remove a negatively charged substance from a liquid by binding thereof. Each ligand comprises a positively charged nitrogen and a thioether linkage at a distance of 1-7 atoms from said charged nitrogen. The
5 desired substances, such as cells, parts of cells and substances comprising peptide structures are adsorbed at salt concentrations in the region of 0.25M NaCl.

Finally, US 6,702,943 (Johansson et al) discloses a method for removal of a target
10 substance from a liquid by adsorption thereof to a matrix carrying a plurality of ligands comprising anion-exchanging groups and a hydrophobic structure. More specifically, the ligands contain an aromatic ring in the proximity of the positively charged anion-exchanging groups. The desired substances are stated to be cells, parts of cells and substances comprising peptide structures. The ligands disclosed are denoted "high salt
15 ligands" due to their capability of adsorbing target substances at high concentrations of salt such as 0.25M NaCl.

However, to optimise a process related to the purification of a specific target molecule, unique operating conditions will be required, and the best separation matrix will vary
20 from case to case. For example, in the biotech industry, specific processes need to be designed for the purification of peptides and proteins; nucleic acids; virus etc. Further, in the purification of antibodies, the type of antibody will decide for the choice of separation matrix. Thus, there is still a need in this field of alternative separation matrices to provide a broad spectrum of choices for the purification of the many new
25 products that are constantly developed.

Brief description of the invention

One aspect of the present invention is to provide a novel ligand which is useful in the separation of antibodies from other components of a liquid.

A specific aspect of the invention is to provide such a ligand, which is capable of adsorbing contaminating proteins but not the target antibody.

Further aspects and advantages of the invention will appear from the detailed description that follows.

Brief description of the drawings

Figure 1 is an illustrative chromatography ligand according to the invention, namely N-benzyl-N-methyl ethanolamine coupled to a support via its amine.

Figure 2 shows a chromatogram of separation of monoclonal antibody on a separation matrix comprising N-benzyl-N-methyl ethanolamine ligands immobilised onto Sepharose™ 6 FF; and for reference, the strong anion-exchanger Q Sepharose™ FF, as described below.

Figure 3 a) and b) show the results of chromatography carried out on ligand prototypes with a mixture of mAb1-rProtein A.

Figure 4 a)-c) show the results of analytical size exclusion chromatography (SEC) on sample with MAb 1, 1% rPrA and pooled flow-through and eluate fractions from the chromatographic runs in figure 3.

Definitions

The terms “antibody” and “immunoglobulin” are used interchangeably in the present specification.

The term “separation matrix” is used herein to denote a material comprised of a support to which one or more ligands comprising functional groups have been coupled. The term “resin” is sometimes used for a separation matrix in this field.

The term “multi-modal” separation matrix refers to a matrix capable of providing at least two different, but co-operative, sites which interact with the compound to be bound. For example, one of these sites may give an attractive type of charge-charge interaction between the ligand and the substance of interest. The other site may give electron acceptor-donor interaction and/or hydrophobic and/or hydrophilic interactions. Electron donor-acceptor interactions include interactions such as hydrogen-bonding, π - π , cation-

π , charge transfer, dipole-dipole, induced dipole etc. "Multi-modal" separation matrices are also known as "mixed mode" separation matrices.

The term "surface" means herein all external surfaces, and includes in the case of a porous support outer surfaces as well as pore surfaces.

- 5 The term "eluent" is used in its conventional meaning in this field, i.e. a buffer of suitable pH and/or ionic strength to release one or more compounds from a separation matrix.

The term "capture step" refers in the context of liquid chromatography to the initial step of a separation procedure. Most commonly, a capture step includes clarification,
10 concentration, stabilisation and a significant purification from soluble impurities. After the capture step, an intermediate purification may follow, which further reduces remaining amounts of impurities such as host cell proteins, DNA, viruses, endotoxins, nutrients, components of a cell culture medium, such as antifoam agents and antibiotics, and product-related impurities, such as aggregates, misfolded species and aggregates.

- 15 The term "disposable" means herein in the context of chromatography columns and other separation matrices a matrix which is intended for single use, or a limited number of uses. Disposable products are advantageously used to remove contaminants which are harmful even in very small amounts, in which case it is convenient to adsorb said contaminant to the matrix and then discard the matrix. Another situation when
20 disposable products are desired is for sterile processing, in which case the matrix is sterile or at least aseptic.

The term "polishing step" refers in the context of liquid chromatography to a final purification step, wherein trace impurities are removed to leave an active, safe product. Impurities removed during the polishing step are often conformers of the target molecule
25 or suspected leakage products.

The term an "Fc-binding protein" means a protein capable of binding to the crystallisable part (Fc) of an antibody and includes e.g. Protein A and Protein G, or any fragment or fusion protein thereof that has maintained said binding property.

Detailed description of the invention

In a first aspect, the present invention is a chromatography ligand comprising an aromatic ethanolamine. The ligand according to the invention is especially useful in the purification of antibodies, as will be discussed in more detail below.

In a first embodiment, the present ligand is defined by the following formula



wherein

R_1 is a substituted or non-substituted aromatic ring system, such as a phenyl group;

R_2 is a hydrocarbon chain comprising 0-4 carbon atoms;

R_3 is a hydrocarbon chain comprising 1-3 carbon atoms;

R_4 is a hydrocarbon chain comprising 1-5 carbon atoms; and

R_5 is OH or H.

As appears from the above, the group R_1 is connected to the amine via a carbon chain R_2 that may comprise no carbon atoms, i.e. constitute a bond between R_1 and the amine; or 1-4 carbon atoms, such as 2-3 carbon atoms, which are optionally substituted. The carbon chain R_4 connecting the amine with the R_5 may comprise 1-5 carbon atoms, such as 2-4 carbon atoms, which are optionally substituted. R_3 of the amine may comprise 1-3 carbon atoms, such as 2 carbon atoms, which are optionally substituted.

The aromatic ring system R_1 may comprise one or more substituted or non-substituted phenyl groups, provided the substitution(s) do not impair the binding properties of the ligand to any substantial extent. Thus, R_1 may comprise one or more aromatic rings, for instance a phenylene, a biphenylene or a naphthylene structure and other aromatic ring systems. Aromatic rings may be heterocyclic, i.e. contain one or more nitrogen, oxygen or sulphur atoms, for instance a pyridine, pyrimidine, pyrrole, imidazole, thiophene, or pyran. Illustrative substituted R_1 groups are selected from the group consisting of hydroxyphenyl (2-, 3- and 4-); 2-benzimidazolyl; methylthioxyphenyl (2-, 3- and 4-); 3-indolyl; 2-hydroxy-5-nitrophenyl; aminophenyl (2-, 3- and 4-); 4-(2-aminoethyl)phenyl;

3,4-dihydroxyphenyl; 4-nitrophenyl; 3-trifluoromethylphenyl; 4-imidazolyl; 4-aminopyridine; 6-aminopyrimidyl; 2-thienyl; 2,4,5-triaminophenyl; 4-aminotriazinyl; and 4-sulphoneamidophenyl.

In an advantageous embodiment, R_1 is non-substituted phenyl. In an alternative
5 embodiment, R_1 is phenyl substituted with one or more OH groups.

Further, one or more of R_1 , R_2 , R_3 , and R_4 may be substituted with any suitable
substituent, as long as the binding properties of the ligand are not impaired to any
substantial extent. For example, if a more hydrophilic ligand is desired, it may comprise
10 one or more hydrophilic groups, such as OH groups. Alternatively, substitution may
increase the hydrophobicity of the ligand, in which case the ligand may comprise one or
more hydrophobic groups, such as alkyl and/or fluorine. Finally, substitution may be
used to introduce one or more additional functionalities, such as charged entities, too
increase the multi-modal character of the ligand. Further, the carbon chains R_2 and R_3
15 may be linear or branched, as long as the branches do not impair the binding properties
of the ligand to any substantial extent.

In a specific embodiment of the present ligand, R_2 is $-\text{CH}_2-$. In another embodiment, R_3
is $-\text{CH}_3$. In a further embodiment, R_4 is $-\text{CH}_2-\text{CH}_2-\text{CH}_2-$ or $-\text{CH}_2-\text{CH}_2-$. In yet another
20 embodiment, R_1 is non-substituted phenyl.

Thus, in an advantageous embodiment, the ligand according to the invention comprises
N-benzyl-N-methyl ethanol amine (BMEA). In an alternative embodiment, the ligand is
N,N-dimethylbenzylamine.

25 The ligand according to the invention is easily synthesised by the skilled person in this
field using standard methods in organic chemistry.

A further aspect of the invention is a method of preparing a separation matrix, which
method comprises immobilising a plurality of ligands as described above to a support. In
30 order to provide a matrix suitable for single use especially in the medical or diagnostic
field, the separation matrix prepared according to the invention is also sterilised in a

subsequent step. Thus, in one embodiment, the method comprises preparing a matrix as described above; providing the matrix so prepared in a column; and sterilizing the matrix so prepared. Sterilization is easily carried out under suitable conditions by the skilled person in this field, such as by heat treatment; radiation; or any other conventionally used method.

As appears from the formula above, in its non-immobilised state, the ligand according to the invention comprises a tertiary amine which will constitute a suitable handle for coupling the thereof to a support, thus creating a coupled ligand which comprises a quaternary amine and a phenyl group. Consequently, as immobilized, the ligand according to the invention is considered to be a multi-modal anion exchange ligand, since in addition to the positively charged quaternary amine group it also comprises the aromatic ring structure which is hydrophobic. Methods for immobilisation of ligands to porous or non-porous surfaces are well known in this field; see e.g. Immobilized Affinity Ligand Techniques, Hermanson et al, Greg T. Hermanson, A. Krishna Mallia and Paul K. Smith, Academic Press, INC, 1992. In one embodiment, the ligands density at the surface of the support is in a range close to what is commonly used for conventional ion-exchange matrices.

In an advantageous embodiment, the coupling of the ligand to the support is provided by introducing a linker between the support and linker. The coupling may be carried out following any conventional covalent coupling methodology such as by use of epichlorohydrin; epibromohydrin; allyl-glycidylether; bis-epoxides such as butanedioldiglycidylether; halogen-substituted aliphatic substances such as di-chloropropanol; and divinyl sulfone. These methods are all well known in the field and easily carried out by the skilled person.

In a specific embodiment, the ligand according to the invention is coupled to the support via a longer linker molecule, also known as extender. Extenders are well known in this field, and commonly used to increase sterically the distance between ligand and support. Extenders are sometimes denoted tentacles or flexible arms, for a more detailed

description of possible chemical structures, see e.g. US 6,428,707, which is hereby included herein via reference. In brief, the extender may be in the form of a polymer such as a homo- or a copolymer. Hydrophilic polymeric extenders may be of synthetic origin, i.e. with a synthetic skeleton, or of biological origin, i.e. a biopolymer with a naturally occurring skeleton. Typical synthetic polymers are polyvinyl alcohols, polyacryl- and polymethacrylamides, polyvinyl ethers etc. Typical biopolymers are polysaccharides, such as starch, cellulose, dextran, agarose. ...

The support may be made from an organic or inorganic material, and may be porous or non-porous. In one embodiment, the support is prepared from a native polymer, such as cross-linked carbohydrate material, e.g. agarose, agar, cellulose, dextran, chitosan, konjac, carrageenan, gellan, alginate, pectin, starch, etc. The native polymer supports are easily prepared and optionally cross-linked according to standard methods, such as inverse suspension gelation (S Hjertén: *Biochim Biophys Acta* 79(2), 393-398 (1964)). In an especially advantageous embodiment, the support is a kind of relatively rigid but porous agarose, which is prepared by a method that enhances its flow properties, see e.g. US 6,602,990 (Berg) or SE 0402322-2 (Berg et al.). In an alternative embodiment, the support is prepared from a synthetic polymer or copolymer, such as cross-linked synthetic polymers, e.g. styrene or styrene derivatives, divinylbenzene, acrylamides, acrylate esters, methacrylate esters, vinyl esters, vinyl amides etc. Such synthetic polymers are easily prepared and optionally cross-linked according to standard methods, see e.g. "Styrene based polymer supports developed by suspension polymerization" (R Arshady: *Chimica e L'Industria* 70(9), 70-75 (1988)). Native or synthetic polymer supports are also available from commercial sources, such as GE Healthcare, Uppsala, Sweden, for example in the form of porous particles. In yet an alternative embodiment, the support is prepared from an inorganic polymer, such as silica. Inorganic porous and non-porous supports are well known in this field and easily prepared according to standard methods.

Suitable particle sizes of the present separation matrix may be in the diameter range of 5-500 μm , such as 10-100 μm , e.g. 20-80 μm . In the case of essentially spherical particles,

the average particle size may be in the range of 5-1000 μm , such as 10-500. In a specific embodiment, the average particle size is in the range of 10-200 μm . The skilled person in this field can easily choose the suitable particle size and porosity depending on the process to be used. For example, for a large scale process, for economical reasons, a more porous but rigid support may be preferred to allow processing of large volumes, especially for the capture step. In chromatography, process parameters such as the size and the shape of the column will affect the choice. In an expanded bed process, the matrix commonly contains high density fillers, preferably stainless steel fillers. For other processes other criteria may affect the nature of the matrix.

Thus, a second aspect of the present invention is a separation matrix which comprises the ligands described above coupled to a support. As understood by the skilled person in this field, each support will commonly comprise a plurality of ligands. In a specific embodiment, the support comprise a ligand as described above in combination with a second kind of ligand, wherein the ligand according to the invention is present to at least about 30%, preferably at least about 50%, more preferably at least about 70% and most preferably at least about 90% of the total ligand amount. Such a combined ligand separation matrix may be designed for a specific case, where an element of further interactions improve its separation properties. The second kind of ligand may comprise one or more charged groups, such as a cation exchanger used to elute compounds by charge repulsion; hydrophobic groups; groups capable of hydrogen-bonding; affinity groups or the like.

In a first embodiment, the matrix according to the invention is in the form of particles, such as essentially spherical, elongated or irregularly formed particles. In a specific embodiment, the separation matrix is dried, such as dried particles which upon use are soaked in liquid to retain their original form. In an illustrative embodiment, such a dried separation matrix is comprised of dried agarose particles. However, the matrix according to the invention may alternatively take any other shape conventionally used in separation, such as monoliths; filters or membranes; capillaries; chips; surfaces; etc.

Consequently, in a second embodiment, the matrix comprises a membranous structure, such as a single membrane, a pile of membranes or a filter.

A third aspect of the present invention is the use of the separation matrix described
5 above. In a first embodiment, the present invention uses a separation matrix as described above in protein purification. In an advantageous embodiment of the present use, the protein is an antibody; an antibody fragment; or a fusion protein comprising an antibody. In another embodiment, the present invention uses a separation matrix as described above in the separation of any other compound, e.g. one selected from the group
10 consisting of polypeptides; nucleic acids, e.g. DNA, RNA or oligonucleotides thereof, plasmids; virus; prions; cells, such as prokaryotic or eukaryotic cells; lipids; carbohydrates; organic molecules, such as small organic molecules; drug targets; diagnostic marker molecules. The use will be discussed in more detail below. In yet another embodiment, the present invention uses a separation matrix as described above
15 as support in cell culture, i.e. to immobilise cells that grow at surfaces. As the skilled person in this field will realise, in the present application, the term separation is used for purification; isolation; and removal of compounds, but it also encompasses identification of a target compound such as for diagnostic purposes.

20 A fourth aspect of the present invention is a method of separation, wherein a desired compound such as an antibody is separated from one or more other compounds of a liquid sample by contacting a mobile phase comprising said liquid sample with a separation matrix as described above. In an advantageous embodiment, the present method is carried out using the principles of liquid chromatography, i.e. by passing a
25 mobile phase over a chromatography column comprising the separation matrix according to the invention. In another alternative embodiment, the present method is carried out using a batch-wise chromatography process, wherein the separation matrix is added to a vessel comprising the liquid sample. In a specific embodiment, the separation matrix added in batch-wise mode comprises dried particles, such as dried agarose particles. In
30 another embodiment, the method is carried out using the principles of expanded bed chromatography i.e. by adding the mobile phase to an expanded bed, such as a fluidised

bed, of a separation matrix which is in the form of essentially spherical particles comprising high density filler.

In a first embodiment of the present method, undesired compounds are adsorbed to the separation matrix while the desired compound, such as the antibodies, remain in the mobile phase without being adsorbed. As understood by the skilled person in this field, the nature and identity of the adsorbed compounds will depend on the origin of the liquid sample. Examples of compounds adsorbed in the embodiment where desired antibodies are not adsorbed are cells and cell debris; proteins and peptides; nucleic acids, such as DNA and RNA; endotoxins, viruses, residues from the culture media etc. In a specific embodiment, the present separation matrix is provided in a chromatography column and the mobile phase is passed across said column by gravity and/or pumping, the antibodies being recovered in the flow-through of the column. Thus, an advantage of this embodiment is that it does not require any elution of the antibody product from the column. Avoiding a specific elution step is attractive from a process point of view, since fewer steps will result in a more rapid purification protocol and consequently reduce the process costs. In addition antibodies are sensitive to certain conditions that would e.g. impair their folding pattern; or degrade them by attacking their peptide bonds. Thus, even though elution conditions for anion-exchangers in general do not involve any extreme chemicals, the change of salt and/or pH may affect the sensitive antibody, the effect varying from species to species depending on the pI, charge distribution etc. Consequently, another advantage of this embodiment is that it avoids adding an eluent and applying eluting conditions to the desired compounds. To obtain the most suitable conditions for adsorption of compounds, the liquid sample is combined with a suitable buffer or other liquid to provide a mobile phase. The present embodiment is advantageously run under conditions conventional for anion-exchange chromatography, which commonly involves adsorption at a relatively low salt concentration. Thus, in one embodiment of the present method, the conductivity of the mobile phase is in the range of 0-25, such as 10-15 mS/cm. In one embodiment, the pH of the mobile phase is about 5-6. If it is desired to subsequently release the adsorbed compounds, e.g. for re-use of the matrix, elution may be carried out at a higher salt concentration, e.g. by use of an

increasing salt gradient. The pH value may also or alternatively be shifted, e.g. be a decreasing pH gradient, to elute adsorbed compounds.

In a second and alternative embodiment of the present method, the desired compounds are adsorbed to the matrix as in conventional liquid chromatography. The matrix may then be reused after selective elution of the product. Elution is easily performed by passing an appropriate buffer over the column. If required, one or more washing steps may be applied before or between any such passage(s). In one embodiment, the operating conditions of this embodiment are as in conventional ion exchange, i.e. adsorption using a mobile phase having low conductivity and elution by using a high conductivity buffer, as discussed above. The skilled person in this field can easily tune the conditions by testing different conditions and analyse the adsorbed compound(s) and flow-through. In a specific embodiment, the desired compounds are antibodies.

Choosing between the first and the second embodiment above, the skilled person in this field can easily adapt the conditions to adsorb a specific compound, advantageously by control of the pH and/or conductivity. For example, in the separation of antibodies, different classes of antibodies have different charges and charge distribution patterns, which together with the purpose of the separation will decide if it is more preferable to adsorb the antibodies or to let them pass the column without being adsorbed.

The antibodies separated according to one embodiment of the present invention may originate from any well known source, such as cells cultured at a surface or from batch-wise or continuous cell culture in fermentation tanks or vessels. Thus, in one embodiment, the liquid is a supernatant obtained from cell fermentation. Examples of compounds that antibodies need to be separated from are then proteins, DNA, viruses, endotoxins, nutrients, components of a cell culture medium, such as antifoam agents and antibiotics, and product-related impurities, such as misfolded species and aggregates.

The step of contact between the mobile phase and the present separation matrix, i.e. the adsorption step, may be preceded by a step of mechanical filtration, centrifugation and/or chromatography. For example, if the liquid sample is a fermentation broth, it is

advantageous to mechanically remove cell debris, whole cells and other relatively large components before the step using the present matrix.

In one embodiment, the present method constitutes the capture step of a purification protocol. In a specific embodiment, the liquid sample is a crude feed which is filtrated before contact with the chromatography matrix according to the invention.

Consequently, this embodiment would still constitute a capture step, even though the liquid sample has been prepurified by mechanical means. As is well known, the host cells that produce antibodies will also comprise a number of other proteins commonly known as host cell proteins (HCP). Such HCPs include enzymes, such as proteases, and other proteins produced by the host cells. Thus, in one embodiment, substantially all host cell proteins of the liquid sample are removed by the present method, such as by adsorption to the separation matrix.

In alternative embodiments, the present method is used as a second, third or even fourth chromatography step in a cleaning protocol, such as an intermediate purification or polishing step. Thus, in one embodiment, the mobile phase applied to the present separation matrix comprises an antibody-containing eluate from a separation matrix. In one embodiment, the liquid sample is an eluate from a preceding affinity chromatography matrix. In an advantageous embodiment, the separation matrix from which the eluate is obtained comprises one or more Fc-binding protein ligands, such as Protein A ligands. The term protein A ligands includes in this context native as well as recombinant protein A, or functional fragments thereof. In this context, the term "functional" fragment means a fragment that has retained the original binding properties of the protein. Such affinity matrices are commercially available, such as MabSelect™ from GE Healthcare. Consequently, in this embodiment, the removed, preferably adsorbed compound may be one or more selected from the group that consists of released Protein A; complexes formed between Protein A and antibodies, such as Protein A-MAb complexes, which complexes may comprise a number of antibodies per Protein A molecule, such as 2-4 antibodies complexed with one Protein A molecule; and aggregates of released Protein A or antibodies. As the skilled person in this field will

understand, depending on the specific conditions used in the preceding step, such as affinity chromatography, the eluate may need conditioning by suitable additions or adjustment. Thus, the eluate is combined with a suitable buffer or liquid to provide a mobile phase.

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The present method is useful to separate any monoclonal or polyclonal antibody, such as antibodies originating from mammalian hosts, e.g. mice, rodents, primates and humans, or antibodies originating from hybridomas. In one embodiment, the separated antibodies are human or humanised antibodies. The antibodies may be of any class, i.e. selected from the group that consists of IgA, IgD, IgE, IgG, and IgM. In one embodiment, the antibodies are antibodies capable of binding to Protein A, or Fc-containing antibody fragments or fusion proteins. In a specific embodiment, the antibodies are immunoglobulin G (IgG), such as IgG1. In one embodiment, the present method is used to purify antibodies having a pI in the range of 6-9, such as in the range of 7-8. In a specific embodiment, the pI of the purified antibodies is about 9. In the present context, it is to be understood that the term "antibodies" also includes antibody fragments and any fusion protein that comprises an antibody or an antibody fragment. Thus, the present invention also encompasses the separation of fragments of any one of the above mentioned antibodies as well as fusion proteins comprising such antibodies. In one embodiment, the antibodies are monoclonal antibodies. In a specific embodiment, the antibodies are humanised antibodies.

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As appears from the above, in the present method, a substantially pure fraction of non-adsorbed antibodies is recovered. In this context, the term "substantially pure" is understood to mean that substantially all the non-antibody compounds have been removed. Most advantageously, at least about 80%, such as at least about 95%, i.e. in the interval of 95-100%, such as at least about 98%, i.e. in the interval of 98-100% and preferably at least about 99%, i.e. in the interval of 99-100%, of the total amount of contaminants is removed on the present separation matrix. However, as the skilled person in this field will appreciate, the purity obtained will depend on the concentration of antibody in the liquid sample applied to the separation matrix as well as other

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conditions used. Thus, in one embodiment, the antibodies separated according to the present method are antibodies of therapeutic grade. Thus, the antibodies purified according to the invention are useful in research and also for the preparation of antibody pharmaceuticals, such as MAb drugs. An alternative use of the purified antibodies is for diagnostic use. Further, the purified antibodies are also useful in food products such as food additives for humans. For example, bovine antibodies purified according to the present invention are useful in food products.

In a specific embodiment of the present method, the present separation matrix is provided as a disposable chromatography column or a disposable filter. An advantage of using disposable products in a method for purification of therapeutic compounds such as antibodies is that it enables avoiding cross-contamination between two different processes. Thus, in one embodiment, the present separation matrix is provided as a sterile chromatography column or filter. In one embodiment, the present method is carried out as a batch-wise process, wherein a disposable separation matrix is added to a vessel comprising the liquid from which the antibodies are to be recovered. A suitable time is allowed for target compounds to adsorb to the matrix, after which the liquid phase comprising the antibodies is removed from the vessel. The used matrix may then be disposed of, without releasing the adsorbed compounds, which again may be advantageous from a safety point of view since compounds such as endotoxins and/or certain host cell proteins need not be handled any further. In an alternative embodiment, the present matrix is provided as a disposable product in a chromatography column which is used in a mode wherein the antibodies are adsorbed. In an advantageous embodiment, the column and matrix have been sterilized, allowing the user to purify antibody product under aseptic or even sterile conditions.

In a second aspect, the present invention relates to a kit for the purification of antibodies from one or more other components in a liquid, which kit comprises in separate compartments a chromatography column packed with a separation matrix as described above; one or more buffers; and written instructions. The separation matrix may be as

described above. Said instructions advantageously describe a method as defined above in detail.

Detailed description of the drawings

- 5 Figure 1 shows the prototype ligand N-benzyl-N-methyl ethanolamine as immobilised via the nitrogen atom to a support in the form of a bead. The coupled ligand is shown to the left with the linker schematically drawn; and to the right with an illustrative hydrophilic linker. In the experimental part, the prototype ligand was coupled to the 6% agarose matrix Sepharose™ 6 FF (GE Healthcare, Uppsala, Sweden).
- 10 Figure 2 shows a chromatogram of sample containing 50 mg Mab1 applied to separation matrices comprising ligands of N-benzyl-N-methyl ethanolamine immobilised on Sepharose™ 6 FF (901035A); N,N-dimethylbenzylamine immobilised on Sepharose 6FF; and Q Sepharose™ FF in 25 mM Bis-Tris, 100 mM NaCl (~ 12 mS/cm), pH 6.5. Elution was performed with 25 mM Bis-Tris, 0.5 M NaCl, pH 6.5.
- 15 Figure 3 a) and b) show the results of chromatography carried out on prototypes with mAb1-rProtein A. A-buffer was 25 mM Bis-Tris, 50 mM NaCl, pH 6.0. The conductivity was approximately 7 mS/cm. B-buffer, 0.5 M Na-Acetate, pH 4.0, was used for elution. Flow rate was 0.5 mL/min (150 cm/h). Sample was 10 mg mAb1, 0.10 mg rPrA at a concentration of 4 mg/ml mAb1 and 1% rProtein A (w/w). 3a) ref. Q
- 20 Sepharose™ FF; and b) N-benzyl-N-methylethanolamine, 146 µmol/mL (901035A). Figure 4 a)-c) show the results of analytical size exclusion chromatography (SEC) on sample with MAb 1, 1% rPrA and pooled flow-through and eluate fractions from the chromatographic runs in figure 3. The blue curve is flow-through (FT) fractions and the red is eluate. More specifically, Figure 4a) shows a sample of 4 mg/mL mAb1, 0.04
- 25 mg/mL rPrA giving 1% (w/w); 4b) shows FT and eluate from fig. 3a) Q Sepharose™ FF; and 4c) shows FT and eluate from fig. 3b) N-benzyl-N-methylethanolamine, 146 µmol/mL (901035A).

EXPERIMENTAL PART

The present examples are provided for illustrative purposes only, and should not be interpreted in any way as limiting the scope of the invention as defined by the appended claims.

Example 1: Preparation of separation matrix according to the invention

Preparation of BMEA Sepharose Fast Flow

One embodiment of the method of preparing a separation matrix according to the invention is shown below, starting from a crosslinked agarose gel (Sepharose™ 6 Fast Flow, GE Healthcare, Uppsala, Sweden).

A. Introduction of allyl group on the matrix

Sepharose 6 Fast Flow was activated with allyl glycidyl ether as follows: 100 ml of Sepharose 6 Fast Flow was suction dried, mixed with 0.3 g of NaBH₄, 12 g of Na₂SO₄ and 35 ml of 50% aqueous solution of NaOH. The mixture was stirred for 1 hour at 50°C. After addition of 100 ml of allyl glycidyl ether the suspension was left at 50°C under vigorous stirring for an additional 16 hours. After filtration of the mixture, the gel was washed successively, with 500 ml distilled water, 500 ml ethanol, 200 ml distilled water 200 ml 0.2 M acetic acid and, 500 ml distilled water.

Titration gave a degree of substitution of 0.22 mmol of allyl/ml of gel.

B. Activation of allyl Sepharose 6 Fast Flow via bromination

Bromine was added to a stirred suspension of 50 ml of allyl activated Sepharose 6 Fast Flow (0.22 mmol allyl groups/ml drained gel), 1 g of sodium acetate and 15 ml of distilled water, until a persistent yellow colour was obtained. Sodium formate was then added until the suspension was fully decolourised. The reaction mixture was filtered and the gel washed with 500 ml of distilled water. The activated gel was then directly transferred to a reaction vessel and further reacted with N-benzyl-N-methylethanolamine.

C. Introduction of BMEA (N-benzyl-N-methylethanolamine) groups on the activated matrix

The amine groups were introduced on the matrix directly via the nitrogen atom of the amine groups. In a typical procedure, the coupling to the matrix was realised via
5 bromination of the allyl group and nucleophilic substitution under basic conditions. 25 ml of bromine activated gel (0.22 mmol allyl groups/ml drained gel) was transferred to a reaction vial containing a solution of N-benzyl-N-methylethanolamine (16.0 ml). 5 ml of water was added and the pH of the reaction solution was adjusted to 12.0 with sodium hydroxide solution. The reaction was left for 16 hours under stirring at 50°C. After
10 filtration of the reaction mixture the gel was successively washed with 3 x 10 ml of distilled water, 3 x 10 ml aqueous 0.5 HCl and finally 3 x 10 ml of distilled water. BMEA Sepharose Fast Flow gel was obtained with a degree of substitution of 0.15 mmol amines/ml of gel.

Example 2: Purification of antibodies in the flow-through

Example 2A) Disposition

Under non-binding conditions, sample containing approximately 50 mg mAb1 were
20 loaded onto prototype 901035 A (N-benzyl-N-methyl ethanolamine) at approximately 5 and 12 mS/cm. Flow-through fractions (FT) were collected at 5, 10 and 15 column volumes (CV). Fractions from the elution peak were pooled. FT fractions were analysed for HCP and Protein A content.

To confirm that the chromatographic performance was not unique for one particular
25 mAb, the chromatographic runs were repeated using a sample containing mAb2 at pH 6.0 and approximately 12 mS/cm. The performance of the prototype was first evaluated with analytical SEC. Selected fractions were analysed for HCP and Protein A content. After screening the fractions with SEC selected fractions were sent for HCP and Protein
30 A analysis.

To test the rProtein A clearance of the prototype, MAb1 was spiked with 1% (w/w) recombinant Protein A (rPrA). The prototype was injected with a sample volume corresponding to 10 mg MAb1, 1% rProtein A at pH 6.0 and a conductivity of approximately 7 mS/cm. Flow-through and eluate fractions were pooled separately and analysed with SEC.

Materials / Investigated units

Columns and gels were obtained from GE Healthcare, Uppsala, Sweden

HiPrep™ 26/10 Desalting	cat.no. 17-5087-01	CV= 53.09 mL
Tricorn™ 5/50	cat.no. 18-1163-09	CV= 1 mL
HR 5/5™	cat.no. 18-0338-01	CV= 1 mL
Superdex™ 200 10/300 GL,	cat.no. 17-5175-01	CV= 23.56 mL

Instruments

Chromatography systems: ÄKTAExplorer™ 10

Spectrophotometer Spectra MAX plus

Chemicals

All chemicals used were of analytical grade. Water was MilliQ-filtered.

Chromatography media

Q Sepharose™ Fast Flow (FF) (GE Healthcare, Uppsala, Sweden). The ligands of the separation matrices are prototypes as described in Table 1 below.

Table 1: Ligand

Prototype ref.	Ligand	Cl ⁻ capacity (μmol/mL)
901035A	N-benzyl-N-methyl ethanolamine	146

Samples

Two different humanised IgG antibodies, subclass 1, denoted MAb 1 and MAb 2, with an extinction coefficient of 1.46 and 1.50 respectively, were used. Both antibodies were expressed in a CHO cultures and subsequently purified using conventional Protein A affinity chromatography prior to the present experiments.

Buffer exchange was made on a HiPrep™ Desalting column (GE Healthcare, Uppsala, Sweden), equilibrated with the buffer of interest, by injecting an appropriate volume (5-15 mL) with a Superloop™ (GE Healthcare, Uppsala, Sweden). The flow rate was 5 mL/min and fractions of 5 mL were collected. Fractions containing the eluted peak were pooled and the absorbance at 280 nm determined in duplicates, in order to calculate the concentration according to equation 1:

$$A_{280} = \epsilon \cdot C \cdot l \quad (\text{Eqn 1})$$

wherein

A_{280} is the absorbance at 280 nm.

ϵ (mL·mg⁻¹·cm⁻¹) is the extinction coefficient for a particular protein.

C (mg/mL) is the concentration of the protein.

l (cm) is the path length.

Size exclusion chromatography (SEC) was performed on a Superdex™ 200 10/300 column (GE Healthcare, Uppsala, Sweden) at a flow rate of 0.5 mL/min. The buffer was PBS (phosphate-buffered saline); 10 mM phosphate, 0.137 M NaCl, 2.7 mM KCl, pH 7.4 prepared from tablets (Sigma, P-4417).

Method

Equilibration 2/ 0.1 CV; 2 CV first time use; 0.1 CV between runs

Sample injection 50 µl

Isocratic Elution 1.5 CV

Chromatography on prototypes with mAb

A-buffer was 25 mM Bis-Tris, pH 6.0 or 6.5. Depending on the desired conductivity, approximately 5 or 12 mS/cm, 35 or 100 mM NaCl was included. The elution buffer (B-buffer) was 25 mM Bis-Tris, 0.5 M NaCl, pH 6.5. The flow rate was 0.5 mL/min (150 cm/h).

10	Method:	Equilibration	5 CV	A-buffer
		Sample injection	5-25 mL	sample cont. 20 or 50 mg mAb
		Wash	5 CV	A-buffer
		Gradient Elution	10 CV	0-100% B-buffer
		Elution	10 CV	100 % B-buffer
		Regeneration	5 CV	A-buffer

Chromatography on prototypes with MAb-rProtein A

A-buffer was 25 mM Bis-Tris, pH 6.0. The conductivity was approximately 7 mS/cm by addition of 50 mM NaCl, B-buffer was 0.5 M Na-acetate, pH 4.0. Flow rate was 0.5 mL/min (150 cm/h). Sample concentration was 4 mg/mL MAb 1- 0.04 mg/mL rPrA giving 1% (w/w).

20	Method:	Equilibration	5 CV	A-buffer
		Sample injection	2.5 mL	10 mg MAb, 1% rPrA
		Wash	5 CV	A-buffer
		Gradient Elution	10 CV	0-100% B-buffer
		Elution	10 CV	100 % B-buffer
		Regeneration	5 CV	A-buffer

CIP (cleaning in place)

After each chromatographic run, the prototype and the reference matrix Q Sepharose™

FF were subject to the following CIP procedure;

	30 % isopropanol	5 CV (Column Volumes)
	H ₂ O	5 CV
	1.0 M NaOH	4 CV (incl. 15 min. pause)
	H ₂ O	5 CV
5	A-buffer	5 CV
	H ₂ O	5 CV
	20% EtOH	5 CV

Protein A analysis

10 Selected fractions were mixed with SPA sample diluent in proportions of 800 µl SPA sample diluent + 200 µl sample. After mixing, the fractions were heated on a heating block at 99 °C for 10 minutes, then mixed again. The samples were then analysed for recombinant Protein A.

15 Host Cell Proteins (HCP) analysis

The samples (min. 600 µl) were analysed for HCP content. The lower detection limit is 10 ng/mL.

20 Example 2B) MAb1-containing sample purified on prototype ligands N-benzyl-N-methylethanolamine (901035A)

Sample containing 50 mg MAb1 was applied to N-benzyl-N-methyl ethanolamine immobilised on Sepharose™ 6 FF (901035A) prepared as described in Example 1 above and the reference matrix Q Sepharose™ FF in 25 mM Bis-Tris, 100 mM NaCl (~ 12
25 mS/cm), pH 6.5. Elution was carried out with 25 mM Bis-Tris, 0.5 M NaCl, pH 6.5.

The chromatograms of example 2 are shown in Figure 2, which shows the prototype N-benzyl-N-methyl ethanolamine Sepharose™ 6 FF (901035A) compared to Q Sepharose™ FF. Flow-through (FT) fractions selected for analysis are indicated with
30 arrows. The results for HCP and Protein A clearance shown in tables 2 and 3 below reveal that the prototype is superior to Q Sepharose™ FF in that respect.

Table 2: Results from HCP analysis

Column	pH	Start (ng/mL)	FT1 (ng/mL)	FT2 (ng/mL)	FT3 (ng/mL)
Q Sepharose™ FF (ref)	6.5	890	160	200	180
N-benzyl-N-methylethanolamine, 146 µmol/mL (901035A)	6.5	890	10	20	35

Table 3: Results from PrA analysis

Column	pH	Start (ng/mL)	FT1 (ng/mL)	FT2 (ng/mL)	FT3 (ng/mL)
Q Sepharose™ FF (ref)	6.5	0.40	0.69	0.46	0.31
N-benzyl-N-methylethanolamine, 146 µmol/mL (901035A)	6.5	0.40	0	0	0

Example 3:**Purification of MAb1 in the flow through from a sample comprising MAb1 and recombinant Protein A (rPrA) on prototype ligand N-benzyl-N-methylethanolamine**

In this example, chromatography on prototypes with a sample containing mAb1-rProtein A was performed. A-buffer was 25 mM Bis-Tris, 50 mM NaCl, pH 6.0. The conductivity was approximately 7 mS/cm. B-buffer was 0.5 M Na-Acetate, pH 4.0. The flow rate was 0.5 mL/min (150 cm/h). Sample was 10 mg mAb1, 0.10 mg rPrA at a concentration of 4 mg/ml mAb1 and 1% rProtein A (w/w). The results are shown in Figure 3.

Finally, analytical SEC on sample with mAb1, 1% rPrA and pooled flow-through and eluate fractions from the chromatographic runs in figure 4 were performed. The results are shown in Figure 4. In Figure 4a, the shaded peak is the complex of MAb1-Protein A. The blue curve is the flow-through (FT) fractions and the red is the eluate.

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Example 4: Adsorption mode

4A) Disposition

To test the selectivity of BMEA Sepharose Fast Flow (BMEA; N-benzyl-N-methylethanolamine) in adsorptive mode the retention time of human IgG and eight different proteins was tested. The results were compared to a commercially available anion exchanger Q Sepharose Fast Flow. The principle of the test method was that proteins were injected into an HR5/5 column (containing the BMEA ligands immobilised on Sepharose™ Fast Flow) equilibrated with the A-buffer (containing piperazine as buffer component). A salt gradient was used for elution of the proteins (see method below).

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Materials / Investigated units

Columns and Q Sepharose Fast Flow were obtained from GE Healthcare, Uppsala, Sweden.

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HR 5/5™: cat.no. 18-0338-01 Column Volume (CV= 1 mL)

Instrument

Chromatography systems: ÄKTAEexplorer™ 10

Chemicals and samples

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The proteins, ovalbumin, β -lactoglobulin, bovine serum albumin, α -lactalbumin, myoglobin, lactoferrin, ribonuclease A and cytochrome C were purchased from Sigma and human IgG (Gammanorm) was purchased from Octapharma. The proteins were dissolved in the A-buffers at a concentration of 1-10 mg/ml. Q Sepharose Fast Flow was obtained from GE Healthcare, Uppsala Sweden. All chemicals used were of analytical grade and the water used was MilliQ-filtered.

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Chromatography

The columns were equilibrated with the A-buffer at a flow rate of 0.6 ml/min before 100 µl of the sample solution were applied. Only one protein at a time was analysed. The proteins were eluted by a linear gradient from buffer A to buffer B with a gradient volume of 21 column volumes (see method below). The buffer A was 25 mM Piperazine, pH 10.0 and buffer B was 25 mM Piperazine, 1.0 M NaCl, pH 10.0. The absorbance at 280 nm was detected during all runs.

Method:

10	Equilibration:	5 CV of A-buffer
	Sample injection:	100 µl (ca 0.2 mg protein)
	Gradient:	21 CV 100 % B-buffer
	Equilibration after gradient:	5 CV of A-buffer

15 Results

To document if the BMEA ligand interact selectively with immunoglobulines, human IgG was applied to a 1 ml column (HR 5/5) packed with the new medium. In addition, the proteins ovalbumin, β-lactoglobulin, bovine serum albumin, α-lactalbumin, myoglobin, lactoferrin, ribonuclease A and cytochrome C were also applied. The results were compared with the retention times of the proteins observed for Q Sepharose Fast Flow. Q Sepharose Fast Flow is a strong anion-exchanger and is used as reference anion exchanger since it has the same support matrix (support material, bead size, pore sizes, pore volume, packing procedure etc) and has essentially the same substitution degree (measured as ion exchange capacity). As appears from Table 1 BMEA Sepharose Fast Flow retarded all the investigated proteins more strongly compared with Q Sepharose Fast Flow. Furthermore, IgG was the protein that gave the longest retention time with BMEA Sepharose Fast Flow (Table 1). This reflects a much stronger binding to the BMEA medium than to Q Sepharose Fast Flow. Compared to Q Sepharose Fast Flow the retention time of IgG increased 27.3 min when BMEA Sepharose Fast Flow was used (Table 1). These results clearly indicate that BMEA Sepharose Fast Flow can be used to capture and elute IgG in a selective way.

Table 1. Retention time (t_r) of different proteins on Q Sepharose Fast Flow and BMEA Sepharose Fast Flow.

Protein	Molecular weight	pI	t_r on Q Sepharose Fast Flow (min)	t_r on BMEA Sepharose Fast Flow (min)	Δt_r (t_r BMEA - t_r Q)
Cytochrome C	12400	9.6	15.3	16.8	1.5
Ribonuclease A	13700	9.4	15.8	22.6	6.8
Lactoferrin	75000	7.9	15.1	19.4	4.3
Myoglobin	17600	7.2	16.1	20.5	4.4
Human IgG	160000		16.5	43.8	27.3
α -Lactalbumin	14400	5.2	24.6	40.3	15.7
Bovine serum albumin	69000	5.1	25.3	32.6	7.3
β -Lactoglobulin	35000	5.1	25.1	37.1	12.0
β -Lactoglobulin	35000	5.1	30.0 ^x	37.1	7.1
Ovalbumin	43500	4.7	21.8	30.8	9.0

5 na = not analyzed, ^x Two peaks were observed

CLAIMS

1. A chromatography ligand defined by the following formula



wherein

- 5 R_1 is a substituted or non-substituted phenyl group;
 R_2 is a hydrocarbon chain comprising 0-4 carbon atoms;
 R_3 is a hydrocarbon chain comprising 1-3 carbon atoms;
 R_4 is a hydrocarbon chain comprising 1-5 carbon atoms; and
 R_5 is OH or H.
- 10 2. A ligand according to claim 1, wherein one or more of R_1 , R_2 , R_3 , and R_4 are substituted with OH.
3. A ligand according to claim 1 or 2, wherein R_1 is a non-substituted phenyl group.
4. A ligand according to any one of the preceding claims, wherein R_2 is $-CH_2-$.
5. A ligand according to any one of the preceding claims, wherein R_3 is $-CH_3$.
- 15 6. A ligand according to any one of the preceding claims, wherein R_4 is $-CH_2-CH_2-$
 CH_2- or $-CH_2-CH_2-$.
7. A ligand according to any one of the preceding claims, which comprises N-benzyl-N-methyl ethanol amine.
8. A method of preparing a separation matrix, which method comprises immobilising a
20 plurality of ligands according to any one of claims 1-7 to a support.
9. A method according to claim 8, wherein the ligand is immobilised via the amine group.
10. A method according to claim 8 or 9, wherein the support is porous.
11. A separation matrix comprising ligands according to any one of claims 1-7 coupled
25 to a support.
12. A matrix according to claim 11, wherein the support comprises particles, such as substantially spherical particles.
13. A separation matrix according to claim 11, wherein the support comprises a membranous structure.

14. A method of manufacturing a chromatography column, which method comprises preparing a matrix as described in claim 11 or 12; providing the matrix so prepared in a column; and sterilizing the column comprising the matrix.
15. A method of manufacturing a separation membrane, which method comprises
5 preparing a membrane as described in claim 13; and sterilizing the membrane.
16. Use of a separation matrix according to any one of claims 11-15 in protein purification.
17. Use according to claim 16, wherein the protein is an antibody; an antibody fragment; or a fusion protein comprising an antibody.
- 10 18. A method of separating one or more antibodies from one or more other compounds in a liquid sample, wherein a mobile phase comprising said antibodies and compound(s) is contacted with a separation matrix according to anyone of claims 11-15.
19. A method according to claim 18, wherein the separation matrix is provided in a chromatography column, the mobile phase is passed across said column by gravity
15 and/or pumping, and the antibodies are recovered in the flow-through of the column.
20. A method according to claim 18 or 19, wherein the liquid sample comprises a supernatant obtained from cell fermentation.
21. A method according to anyone of claims 18-20, wherein the contact with the separation matrix is preceded by a step of mechanical filtration and/or
20 chromatography.
22. A method according to anyone of claims 18-21, wherein the liquid sample comprises a crude feed.
23. A method according to anyone of claims 18-22, wherein the adsorbed compound(s) are host cell proteins and substantially all said proteins are adsorbed to the separation
25 matrix.
24. A kit for the purification of antibodies from one or more other components in a liquid, which kit comprises, in separate compartments, a chromatography column packed with a separation matrix according to any one of claims 11-15; one or more buffers; and written instructions.

25. A disposable chromatography column for the purification of antibodies, which column comprises a separation matrix according to any one of claims 11-15.
26. A column according to claim 25, which has been sterilised.

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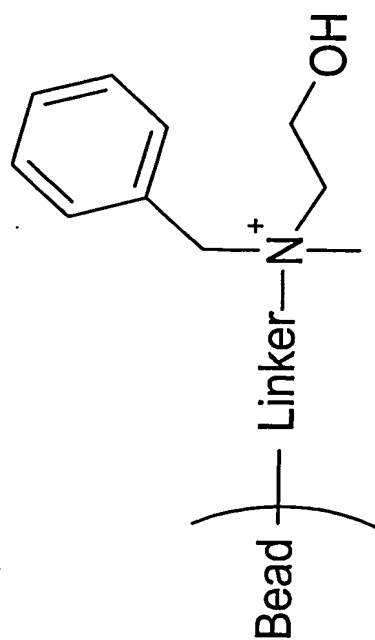
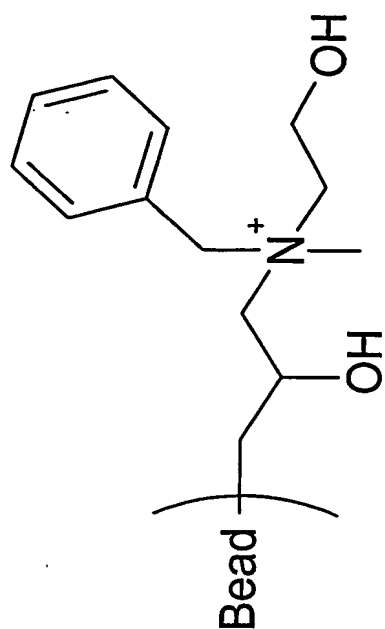
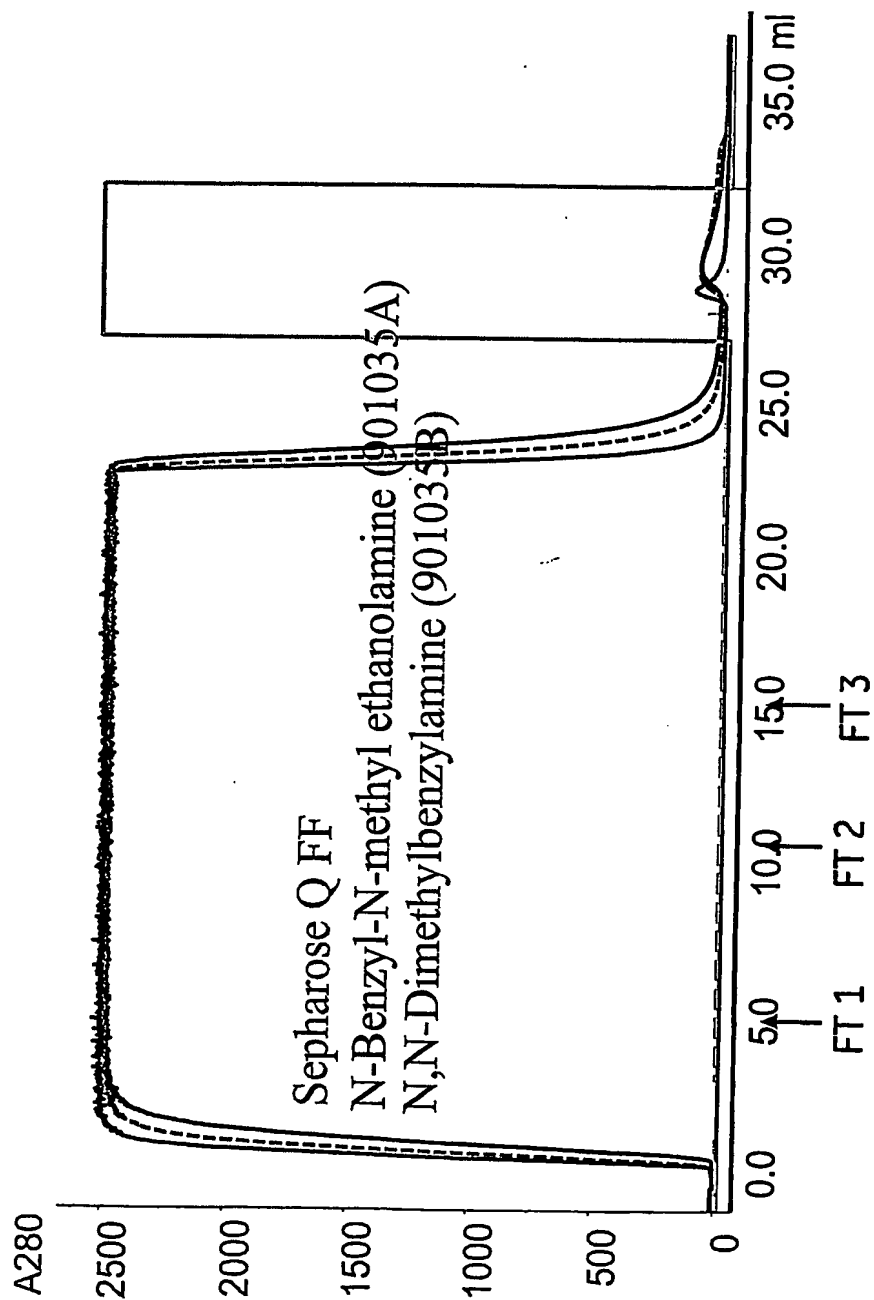


Figure 1

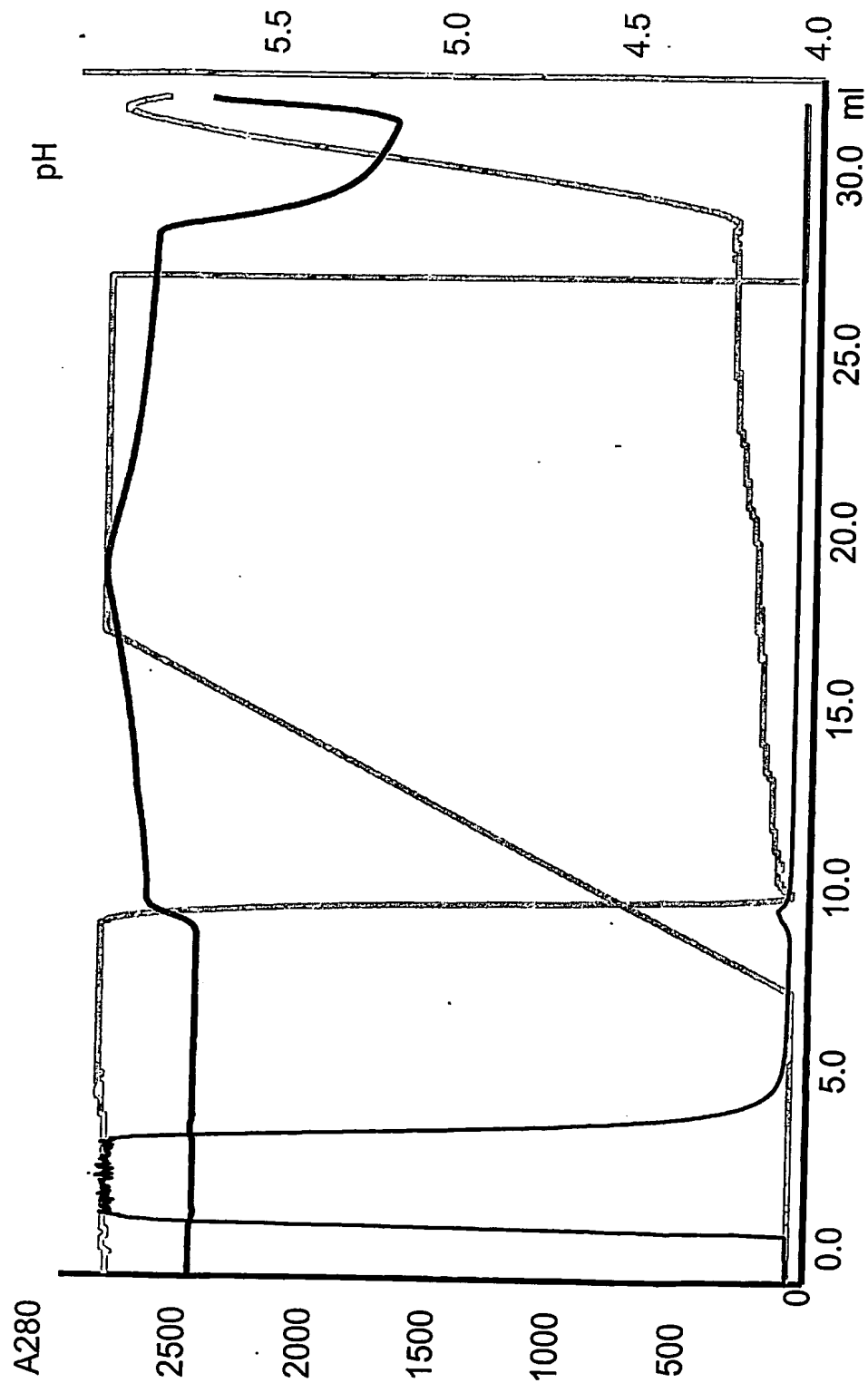
2/7

Figure 2



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Figure 3 a



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Figure 3 b

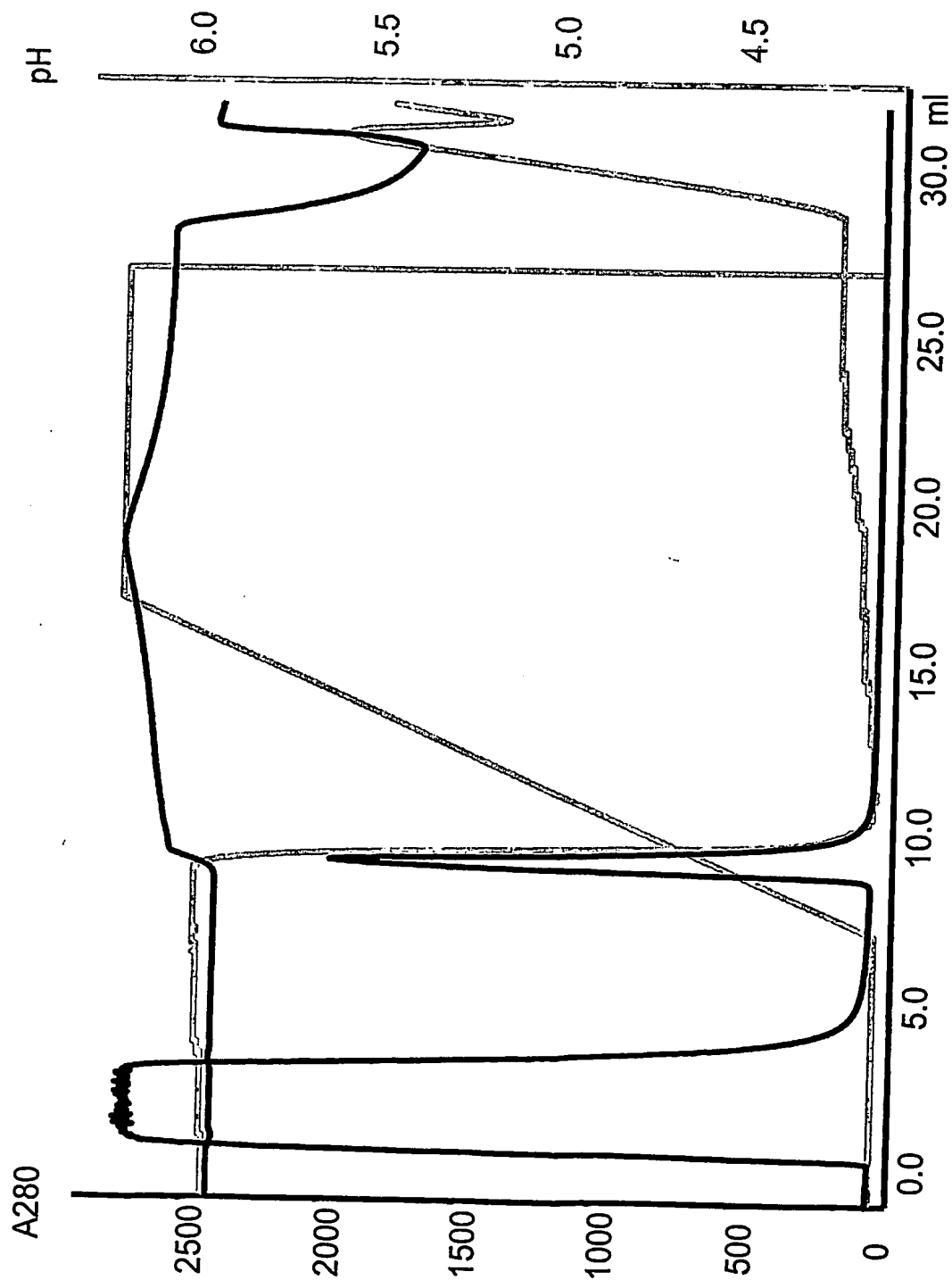


Figure 4 a

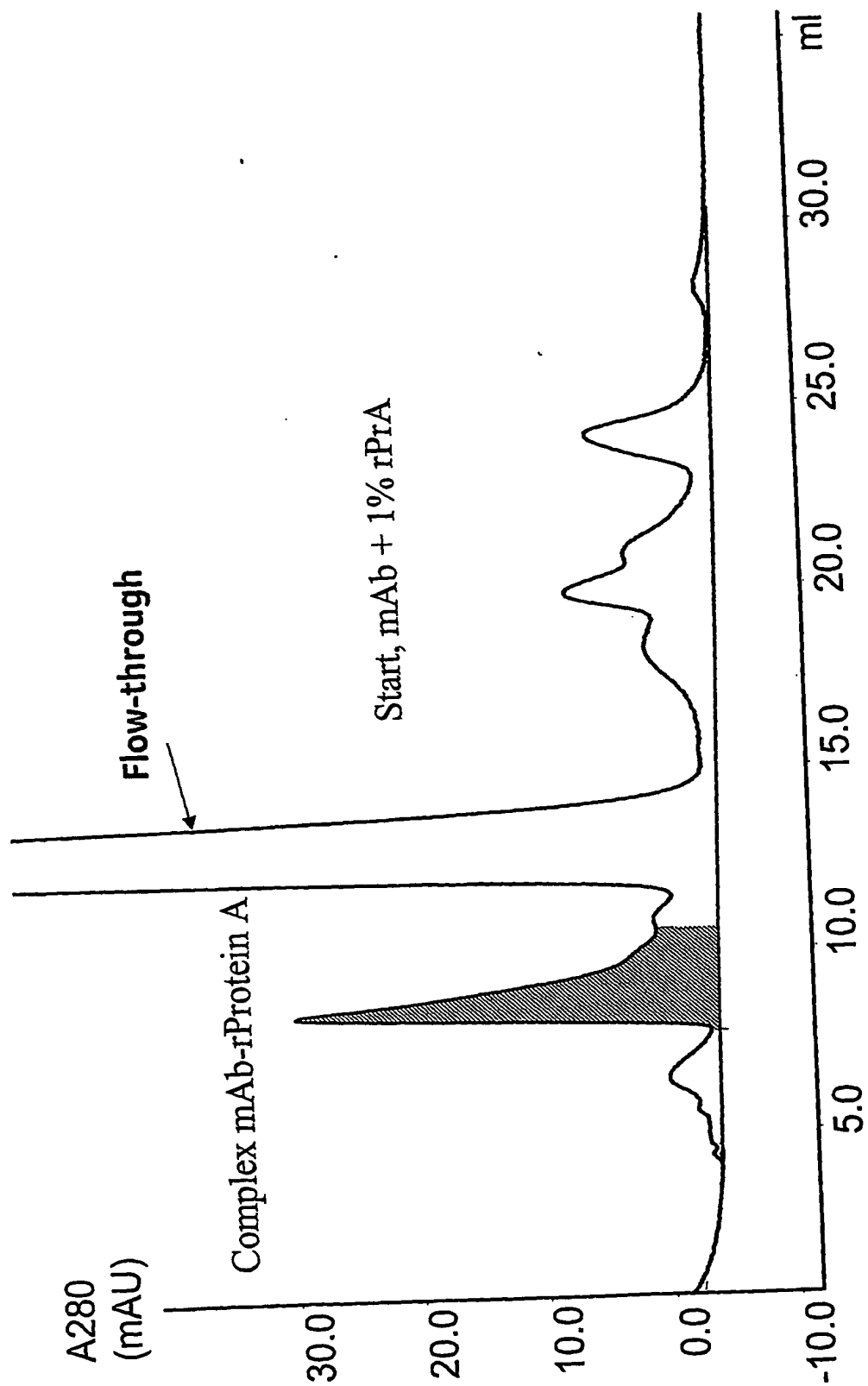
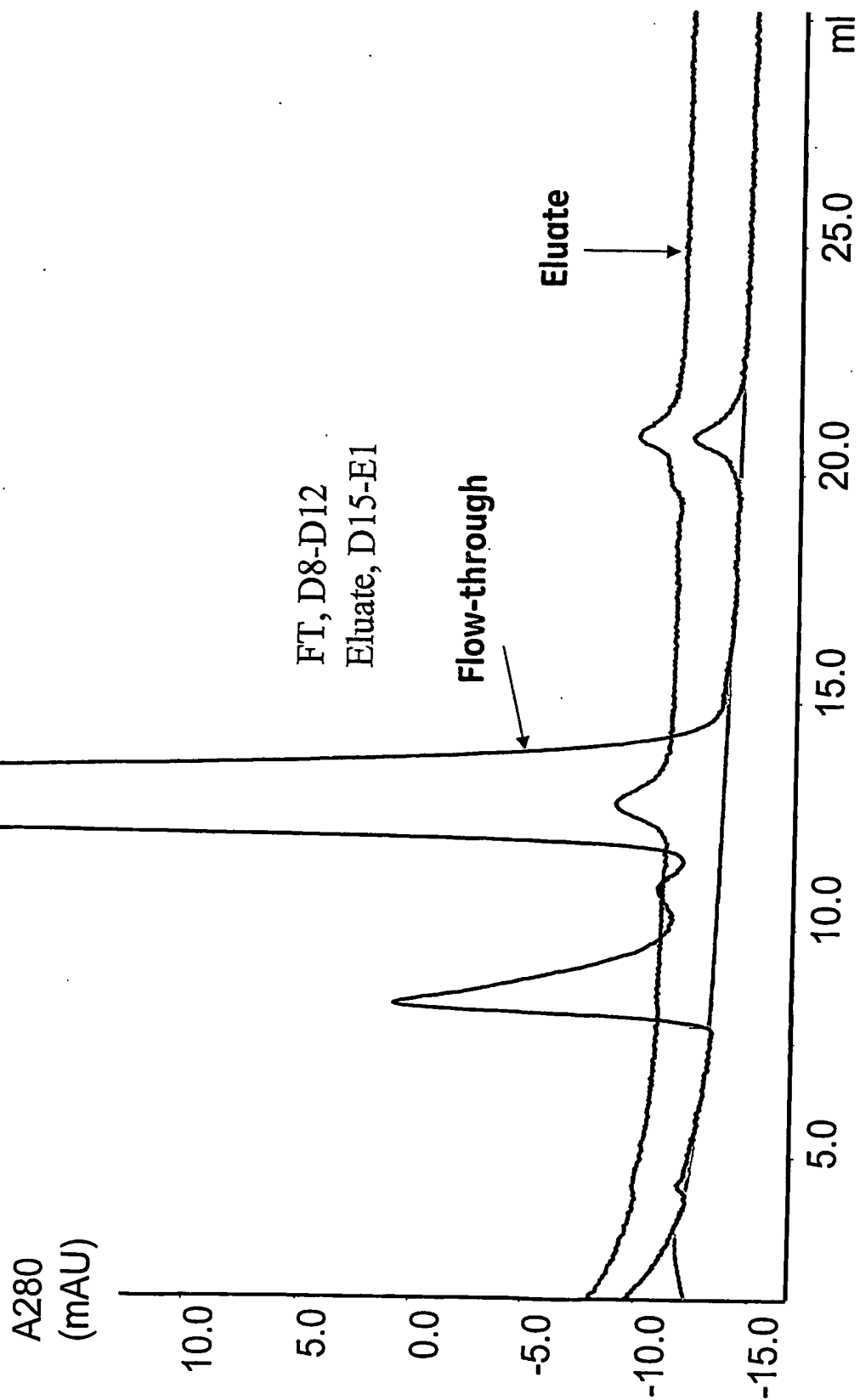
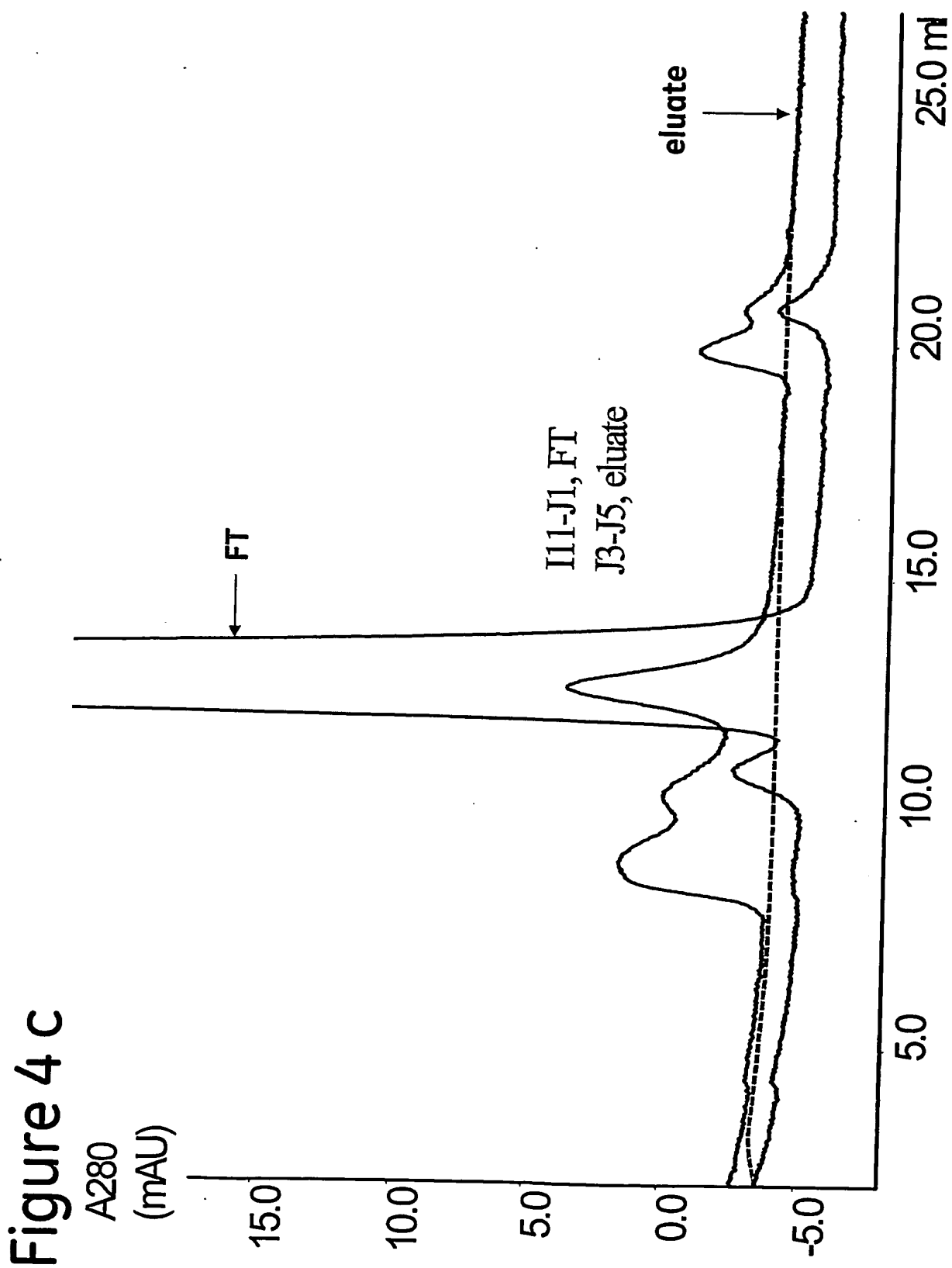


Figure 4 b



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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 2005/001592

A. CLASSIFICATION OF SUBJECT MATTER

IPC: see extra sheet
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: B01J, B01D, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-INTERNAL, WPI DATA, PAJ, BIOSIS, MEDLINE, REGISTRY, CA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HABTEMARIAM, ABRAHA ET AL, "Control of aminophospine chelate ring-opening in Pt(II) and Pd(II) complexes: potential dual-mode anticancer agents", J. Chem. Soc., Dalton Trans., 2001, page 1306 - page 1318, Scheme 2 --	7
X	WO 0138227 A2 (AMERSHAM PHARMACIA BIOTECH AB), 31 May 2001 (31.05.2001) --	8-25
A	WO 2004076485 A1 (LONZA BIOLOGICS PLC.), 10 Sept 2004 (10.09.2004) --	8-25

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

7 February 2006

Date of mailing of the international search report

08-02-2006

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 2005/001592

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>JOHANSSON, BO-LENNART ET AL, "Preparation and characterization of prototypes for multi-modal separation aimed for capture of positively charged biomolecules at high-salt conditions", Journal of chromatography A, 2003, volume 1016, page 35 - page 49</p> <p style="text-align: center;">-- -----</p>	8-25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE05/001592

INTERNATIONAL PATENT CLASSIFICATION (IPC) :

B01J 41/04 (2006.01)

B01D 15/32 (2006.01)

B01D 15/36 (2006.01)

C07K 16/00 (2006.01)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE05/001592

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-6
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Present claims 1-6 and dependent parts of claims 8-26 relate to an extremely large number of possible compounds.

.../...

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Box II.2

In fact, the claims contains so many options that a lack of clarity and conciseness within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claims impossible.

Consequently, the search has been carried out for those parts of the application which appear to be clear and concise, namely the compound according to claims 7 and related methods etc. in claims 8-26.

Moreover, a search for the use of tertiary or quaternary amines in multi-modal chromatography has been performed; however this search can not be regarded as covering all ligands within the scope of claims 1-6.

INTERNATIONAL SEARCH REPORT

Information on patent family members

26/11/2005

International application No.

PCT/SE 2005/001592

WO	0138227	A2	31/05/2001	AU	780286	B	10/03/2005
				AU	782945	B	08/09/2005
				AU	1704401	A	04/06/2001
				AU	2507901	A	04/06/2001
				CA	2389515	A	31/05/2001
				CA	2392200	A	31/05/2001
				EP	1235748	A	04/09/2002
				EP	1235749	A	04/09/2002
				IL	149782	D	00/00/0000
				IL	149783	D	00/00/0000
				JP	2003514664	T	22/04/2003
				JP	2003531713	T	28/10/2003
				SE	9904197	D	00/00/0000
				US	6702943	B	09/03/2004
				US	20040079702	A	29/04/2004
				WO	0138228	A	31/05/2001

WO	2004076485	A1	10/09/2004	AU	2003278327	A	00/00/0000
				AU	2004215653	A	10/09/2004
				CA	2516518	A	10/09/2004
				GB	0304576	D	00/00/0000
				CN	1574540	A	02/02/2005
				JP	2005039993	A	10/02/2005
				US	20040257042	A	23/12/2004